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Kinetics of Reticulocyte Maturity Fractions and Indices and Iron Status During Therapy With Epoetin Beta (Recombinant Human Erythropoietin) in Cardiac Surgery Patients

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We evaluated the changes in reticulocyte maturity fractions and indices, as measured by flow cytometry, during preoperative treatment with recombinant human erythropoietin (epoetin beta) in cardiac surgery patients. A total of 72 patients was enrolled in this double-blind, randomized, placebo-controlled clinical trial and assigned to the two treatment groups (5 × 500 U/kg bodyweight epoetin beta or placebo intravenously over 14 days preoperatively).

Therapy with epoetin beta produced continuous increases in hematocrit/hemoglobin, in the most mature fraction of reticulocytes (LR), and in reticulocyte count. In the first treatment week there were parallel increases in the fraction of most immature reticulocytes (HR) and in the reticulocyte mean cell volume. During the second week of treatment the reticulocyte mean cell hemoglobin content (CHr) decreased, but CHr was independent of all iron parameters, affecting neither the reticulocyte fractions nor the hematocrit/hemoglobin increase. The total preoperative rise in hematocrit correlated with the rises in LR fraction (*P* = 0.0270) and reticulocyte count (*P* = 0.0486) during the first week of treatment. Whereas in the epoetin beta patients the preoperative change in HR fraction showed negative correlations with transferrin saturation at baseline (*P* = 0.0058) and with the preoperative change in iron (*P* = 0.0113), the preoperative change in the LR fraction correlated positively with transferrin at baseline (*P* = 0.0115). Postoperatively, the reticulocyte parameters revealed that the onset of increased stimulation of erythropoiesis did not occur in the placebo patients until the second postoperative day, whereas erythropoietic activity in the epoetin beta patients was much higher during the postoperative period as well, as a result of the preoperative stimulation of erythropoiesis.

The reticulocyte parameters measured by flow cytometry permitted an objective analysis of erythropoietic activity during treatment with epoetin beta and in all patients postoperatively. Further studies in various types of epoetin beta therapy are needed in order to clarify the value of these reticulocyte parameters for identification of iron deficiency and optimization of epoetin beta treatment regimen. Am. J. Hematol. 55:89–96, 1997.

Key words: blood transfusion; erythropoietin; flow cytometry; cardiac surgery; iron metabolism; reticulocyte

INTRODUCTION

Despite the introduction of new techniques and the optimization of existing ones, about 70% of all cardiac operations, and particularly high-risk procedures, still require allogeneic blood transfusions (ABT) [1]. The use of

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recombinant human erythropoietin (rhEPO) is currently being investigated in this particular context as a means of avoiding ABT. This drug has proved effective over many years in the treatment of renal anemia and has been used in orthopedic and cardiac surgery patients during autologous blood donation (ABD) in order to increase the donated blood volume [2–4]. The new ABT-prevention concept of rhEPO treatment without ABD has been successfully applied in cardiac surgery for Jehovah's Witnesses and for patients with contraindications for ABD [5,6]. The evaluation of rhEPO response and the optimization of iron therapy are of importance in optimizing rhEPO therapy in all indications. The introduction of reticulocyte flow cytometry has improved reticulocyte analysis, and the technique also possesses a favorable cost-benefit ratio [7,8]. Preliminary results for these relatively new parameters during the rhEPO treatment of various anemias, as well as the results of our own pilot study, have since been published [9–12]. In a double-blind, placebo-controlled randomized trial, we investigated the changes in reticulocyte maturity fractions and indices and their relationships to iron parameters and to hematocrit in cardiac surgical patients receiving preoperative therapy with rhEPO (epoetin beta). Consequently, the trial provides an evaluation of the hitherto poorly investigated diagnostic value of these parameters in high-dose rhEPO therapy.

PATIENTS AND METHODS

The trial protocol was approved by the Ethics Committee of Charité Hospital, Humboldt University, Berlin. Patients (age 18–80 years) undergoing elective cardiac surgery and with contraindications for ABD, due to their restricted cardiac function (e.g., aortic valve stenosis, coronary left main stenosis, poor left ventricle), were enrolled in the trial. The following exclusion criteria were applied: diastolic blood pressure >100 mm Hg, hematocrit >0.45, convulsions or epilepsy, platelet count $>450 \times 10^9$/l, malignant tumor, acute infections, pregnancy, lactation, or inadequate contraception. Patients were randomly enrolled in the trial 14 days before surgery, having been briefed accordingly and having given their consent, and after a medical history had been taken. The trial medication, 500 U epoetin beta or placebo/kg body weight intravenously was administered 14, 10, 7, 5, and 2 days preoperatively. All patients received daily oral doses of 300 mg Fe^{2+} (iron-glycin-sulphate) during the 14-day treatment phase. The blood picture and reticulocyte maturity fractions and indices were determined on the days of administration of the trial medication, immediately before surgery and 2, 5, and 7 days postoperatively. The iron parameters (iron, ferritin, transferrin, and transferrin saturation) were determined at the start of treatment, immediately before surgery, and on the seventh postoperative day.

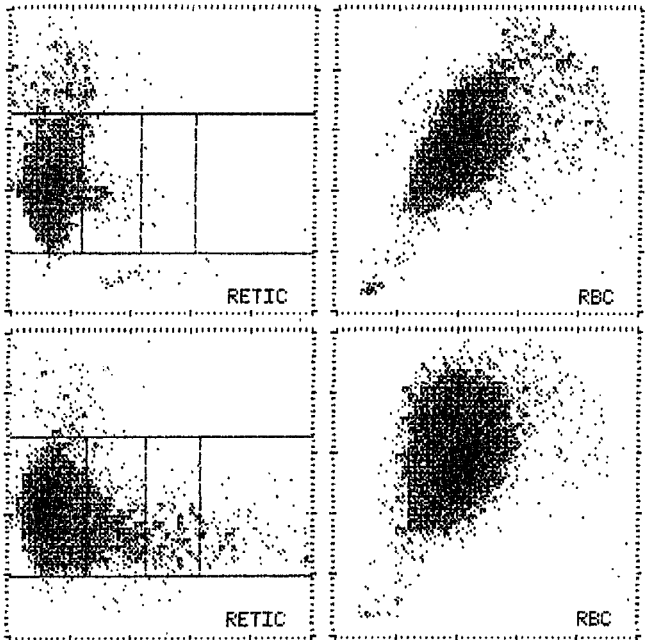

Fig. 1. Reticulocyte histogram (H*3 Technicon) for a patient before (14 days before the operation, above) and after (immediately before the operation, below) 5 × 500 U epoetin beta/kg body weight within 14 days. Left: Plots of absorption intensity (x-axis) vs. red scatter high (y-axis, 5–15°). The two horizontal lines show the range of forward-angle scatter for red cells and reticulocytes. The vertical lines mark the range for red cells and reticulocytes according to intensity of absorption (from left to right: red cells, low absorptioned retics, medium absorptioned retics, high absorptioned retics). Right: Plots of red scatter high (x-axis, 5–15°) and red scatter low (y-axis, 2–3°).

The reticulocyte analysis employed the Technicon H*3 system (Bayer Diagnostics, Munchen, Germany). In the H*3-reticulocyte counting method the reticulocytes are stained with Oxacin-750 (Bayer Diagnostics, Munchen, Germany), a stain that binds to nucleic acids, and are counted by the respective light absorption of each cell as it passes a light beam [7]. The light absorption is proportional to the RNA content of the cells, from which the degree of maturity of the reticulocytes can be derived (Fig. 1). Immature reticulocytes possess more RNA and show higher absorption values than mature cells. The reticulocytes are classified into three different maturity stages depending on their absorption: HR (high absorptioned retics), the most immature fraction; MR (medium absorptioned retics), medium maturity fraction; LR (low absorptioned retics), the most mature fraction. The volume and hemoglobin content of each cell, and thus the reticulocyte mean cell hemoglobin concentration (CHCMr), the reticulocyte mean cell volume (MCVr), and the reticulocyte mean cell hemoglobin content (CHr), were determined by a technique based on the Mie scattered light theory [8].

Continuous laboratory variables at various time points

TABLE I. Demographic and Clinical Characteristics and Baseline Values of Iron Parameters of Patients Included in the Analysis of Efficacy, Undergoing Elective Open-Heart Surgery and Receiving 5 × 500 U/kg Epoetin Beta-Placebo Within 14 Days Preoperatively*

	Median (interquartrilrange) in trial group	
Variable	Epoetin beta (n = 36)	Placebo (n = 36)
Age (years)	55.5 (49.5–60.5)	59.5 (53.5–62.5)
Male/female (no.)	28/8	28/8
Body weight (kg)	77.5 (68.5–91.0)	75.0 (70.0–83.5)
Height (cm)	172 (168–179)	170 (165–175)
Iron at baseline (μmol/l)	12.6 (9.2–17.4)	12.0 (8.9–16.3)
Ferritin at baseline (μg/l)	132.6 (67.9–167.0)	115.4 (44.5–149.4)
Transferrin (g/l)	3.0 (2.8–3.4)	3.2 (2.8–3.4)
Transferrin saturation at baseline (%)	17.2 (13.2–25.3)	17.3 (13.0–23.5)
ABT per patient (ml)	0 (0–0)	300 (0–600)
Total blood loss (ml)	855 (595–1,030)	695 (565–1,050)

*ABT, allogeneic blood transfusions (packed red cells) intra- and postoperatively.

were compared between the two groups by analysis of covariance using the baseline value as a covariate. Univariate and multiple regression analyses (maximum likelihood method, stepwise selection technique) were performed to examine the relationship between the changes in hematocrit/hemoglobin from baseline to day of surgery and the early changes in the various reticulocyte parameters after the start of epoetin beta treatment, as well as various possible prognostic factors at baseline. Furthermore, a regression analysis was performed to determine the relationship between the changes in hematocrit/hemoglobin and the reticulocyte parameters during the preoperative treatment phase on the one hand, and the iron parameters at baseline and their preoperative changes on the other. Similar analyses were performed for the postoperative changes in hematocrit/hemoglobin. Two-tailed tests were used exclusively. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Thirty-six patients were randomly allocated to each group, received trial medication, and were included in the analysis of efficacy. No significant differences were apparent between the groups as regards age, sex ratio, body weight, height, blood loss, or baseline values of all laboratory parameters (Table I). In this trial, ABT was reduced by a factor of 4–5 under treatment with epoetin beta [6].

In the placebo group the hematocrit, reticulocyte count, and various reticulocyte maturity fractions and indices remained unchanged preoperatively (Figs. 2, 3). Epoetin beta therapy produced continuous preoperative increases in the reticulocyte count (Fig. 2a), LR fraction (Fig. 3a), and hematocrit (Fig. 2b)/hemoglobin (Table II). The HR and MR fractions rose sharply in the first few days, followed by a slight decrease, though remaining at a high level until surgery (Table II, Fig. 3b,c). There was a drop in CHCMr and a rise in MCVr during the first treatment week (Fig. 4a,b). Neither parameter showed any relevant change during the second treatment week, whereas the CHr fell during the same week (Fig. 4c). Preoperatively, serum iron, ferritin, and transferrin saturation dropped by 5.2 μmol/l, 67.3 μg/l, and 6.6%, respectively, in the epoetin beta group but increased by 4.7 μmol/l, 29.5 μg/l, and 9.9%, respectively, in the placebo group. In both groups, transferrin remained unchanged.

The univariate and multivariate regression analyses investigating the correlation between hematocrit/hemoglobin, reticulocyte parameters, and iron parameters produced the following results in the epoetin beta patients: the greater the increases in LR fraction (univariate and multivariate: $P = 0.0270$, $r = 0.44$) and in the reticulocyte count (only univariate: $P = 0.0486$, $r = 0.40$) during the first 7 days of treatment, the greater the overall preoperative increase in hematocrit. Age, sex, weight, and height of patients, the changes in the MR and HR fractions, and reticulocyte indices did not significantly affect the subsequent increase in hematocrit/hemoglobin in both groups.

The lower the transferrin saturation at baseline (univariate/multivariate: $r = -0.53$, $P = 0.0058$) and the greater the preoperative iron reduction (multivariate: $r = -0.42$, $P = 0.0113$), the higher the preoperative increase in the HR fraction in the epoetin beta group. The same predictive factors were determined for the preoperative increase in MR fraction (transferrin saturation at baseline-univariate/multivariate: $r = -0.41$, $P = 0.0356$; iron change preoperatively-multivariate: $r = -0.38$, $P = 0.0371$). The higher the transferrin at baseline (univariate and multivariate: $r = 0.49$, $P = 0.0115$) in the epoetin

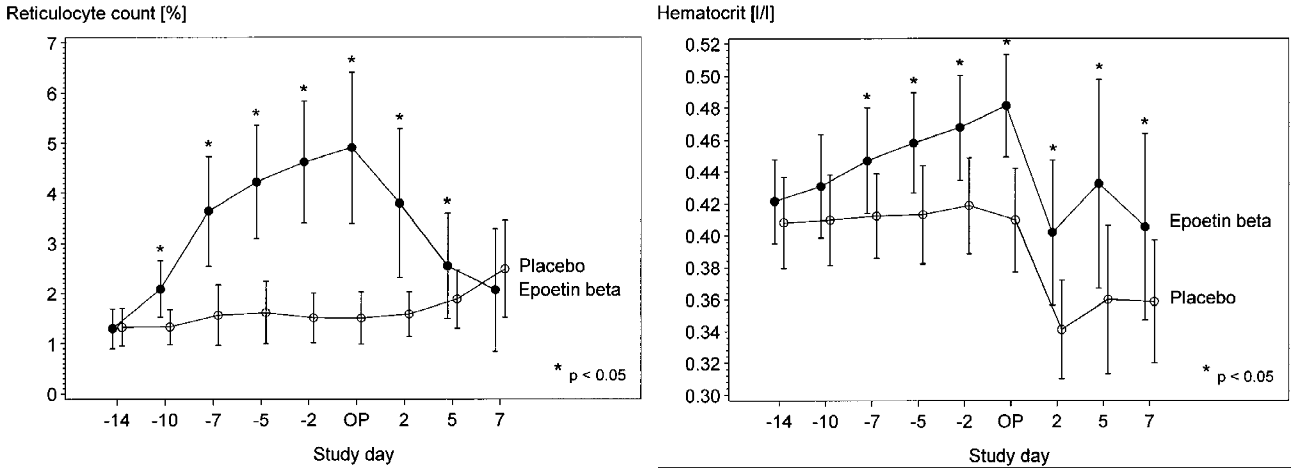

Fig. 2a-b. Perioperative course (mean ± standard deviation) of reticulocyte count (a) and hematocrit (b). *Comparison between both groups at various time points (covariance analysis using the baseline value as a covariate) ($P < 0.05$).

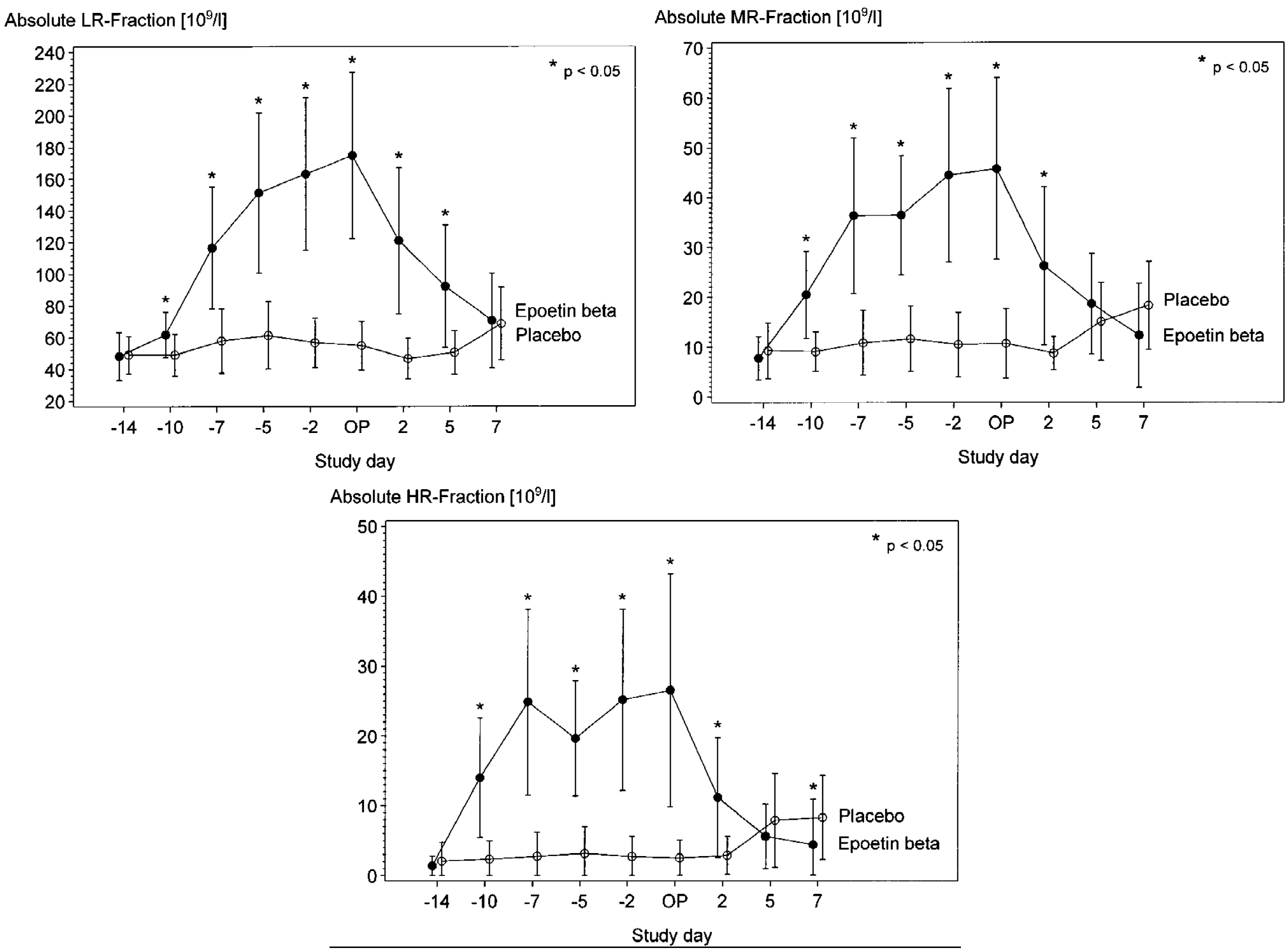

Fig. 3. a-c: Perioperative course (mean ± standard deviation) of absolute counts of low-absorptioned retics (a) (LR), medium-absorptioned retics (b) (MR), and high-absorptioned retics (c) (HR). *Comparison between both groups at various time points (covariance analysis using the baseline value as a covariate) ($P < 0.05$).

TABLE II. Hematocrit, Hemoglobin, Reticulocyte Count, Reticulocyte Fractions, and Indices in Epoetin Beta Patients at Baseline (14 Days Preoperatively) and Immediately Before Surgery (*Day of Surgery Preoperatively*)

	Median (interquartilrange)	
Variable	14 days preoperatively	Day of surgery preoperatively
Hematocrit (l/l)	0.42 (0.41–0.44)	0.49 (0.47–0.51)
Hemoglobin (mmol/l)	8.90 (8.60–9.35)	9.95 (9.40–10.30)
Reticulocyte count (%)	1.27 (1.00–1.58)	4.75 (3.67–6.20)
Absolute HR fraction ($\times 10^9$/l)	0.7 (0.3–2.4)	24.2 (17.2–31.6)
Absolute MR fraction ($\times 10^9$/l)	6.2 (4.4–11.1)	45.0 (33.4–58.5)
Absolute LR fraction ($\times 10^9$/l)	50.5 (38.0–58.8)	164.1 (140.8–221.2)
CHCMr (mmol/l)	17.5 (17.0–18.2)	15.2 (14.7–16.1)
MCVr (fl)	106.7 (103.5–108.0)	119.9 (116.0–123.7)
Chr (fmol)	1.82 (1.78–1.87)	1.75 (1.65–1.88)

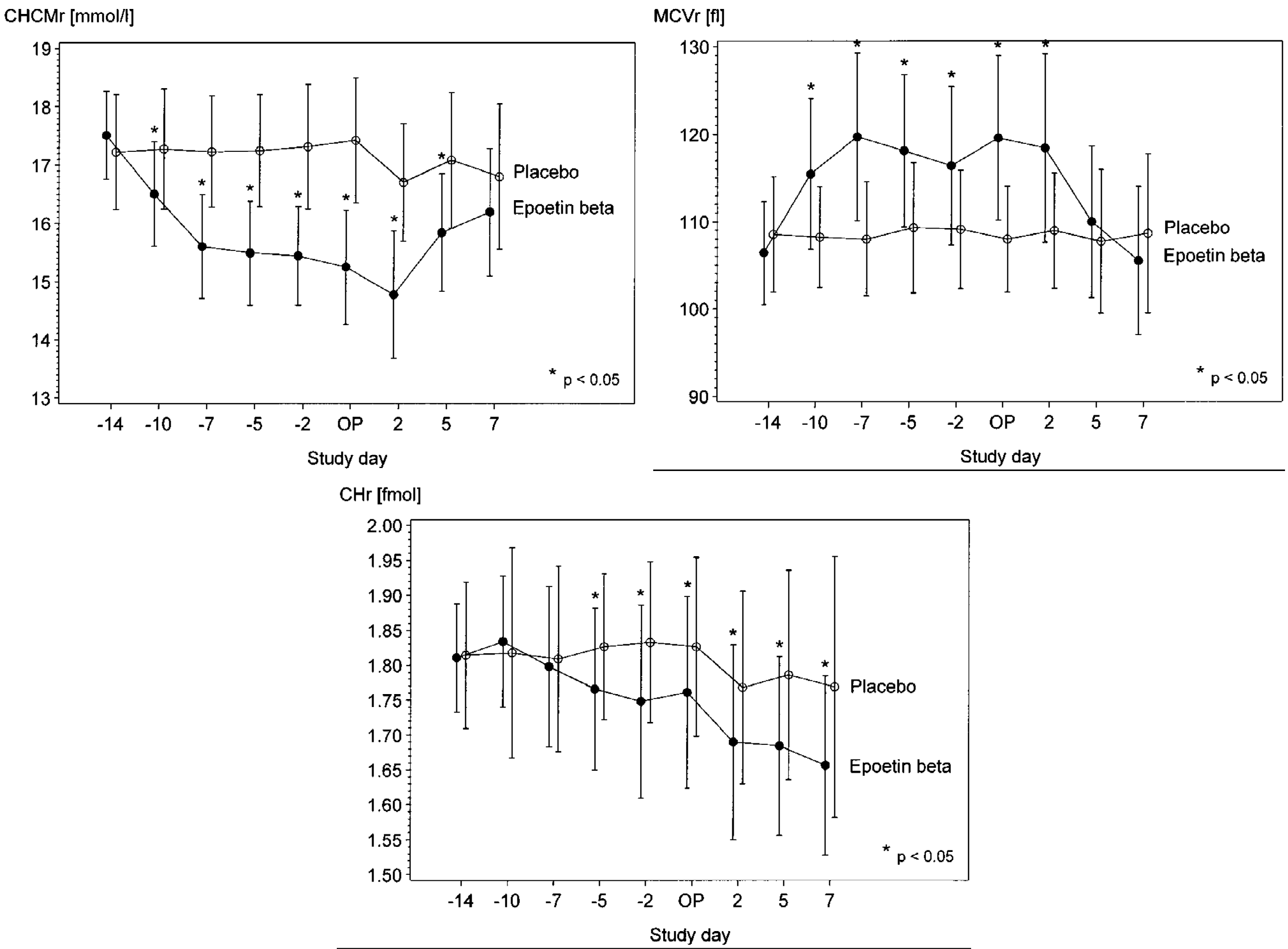

Fig. 4. a-c: Perioperative course (mean ± standard deviation) of reticulocyte mean cell hemoglobin concentration (a) (CHCMr), reticulocyte mean cell volume (b) (MCVr), and reticulocyte cell hemoglobin content (c) (CHr). *Comparison between both groups at various time points (covariance analysis using the baseline value as a covariate) (*$P < 0.05$*).

beta patients, the greater the preoperative rise in LR fraction. In neither group was there any correlation between the reticulocyte indices on the one hand and the reticulocyte fractions and all iron parameters on the other (baseline value, preoperative change). In the placebo group no correlations were obtained from the regression analyses of any of the above parameters.

The reticulocyte count and all reticulocyte fractions

increased in the placebo group and decreased in the epoetin beta patients in the postoperative phase (Figs. 2, 3). In the placebo group the CHCMr and the MCVr remained unchanged postoperatively, whereas the CHr had dropped by 0.06 fmol by the second postoperative day, remaining constant until the end of the trial (Fig. 4a–c). In the epoetin beta group the CHr continued to fall postoperatively. The CHCMr showed a rise while the MCVr dropped, though neither parameter reached its baseline level in the epoetin beta group. The multiple and univariate regression analyses failed to show any significant effect of the immediate preoperative values and of the postoperative changes for reticulocyte fractions and indices on the postoperative course of hematocrit/hemoglobin in both groups. Age, sex, weight, height, intra- and postoperative blood loss, and iron parameters (immediately before surgery and their change postoperatively) showed no correlation with postoperative changes in hematocrit/hemoglobin or the reticulocyte parameters.

DISCUSSION

The continuous increases in reticulocyte count and hematocrit/hemoglobin, and the decrease in storage iron, are evidence of the dose-dependent increase in erythropoiesis in epoetin beta patients. The sharp rise in the HR fraction indicates that a large portion of immature reticulocytes already present in the bone marrow are rapidly released after the start of treatment into the peripheral blood, together with mature reticulocytes. The reticulocyte maturation period of these immature reticulocytes, or ‘‘shift cells,’’ is shortened in the bone marrow and is prolonged in the peripheral blood as a result of epoetin beta therapy [13–15]. An initial proportional increase in HR fraction was also described by Tatsumi et al. [10] (obtained by the fluorescent method) in relation to the rhEPO treatment of renal anemia. They interpreted the rise in HR as an indicator of erythropoietic response to rhEPO. However, since the preoperative increase in hematocrit/hemoglobin in our investigations did not correlate with the rise in HR, the HR rise simply represented the initial response of the reticulocyte pool. The release of immature cells remained almost constant after the tenth treatment day and is the sum of both rhEPO effects on bone-marrow reticulocytes and on the omission of maturation stages of precursor cells. A striking finding was the parallel kinetics plotted for MCVr, CHCMr, and the HR fraction in the preoperative period. The reticulocytes in the HR fraction are larger in volume since the cell volume constantly decreases from the proerythroblast stage onward during erythropoiesis [16]. A connection exists between hemoglobin concentration and DNA synthesis, insofar as above a certain hemoglobin concentration, DNA synthesis and cell division are suppressed. Since rhEPO also stimulates hemoglobin formation, high-dose treatment with the substance may actually initiate such formation and thus bring forward the time of final cell division, resulting in the generation of megareticulocytes [17,18].

The positive correlation between the increases in reticulocyte count and the LR fraction in the first treatment week with the total hematocrit increase is of limited value in determining the point at which the target hematocrit rise was reached. In view of the degree of uncertainty involved in these correlations and the lack of corresponding correlations for hemoglobin increase, any adjustments made to epoetin beta therapy on the basis of these parameters would not be sufficiently justified.

Like us, Brugnara et al. [9] observed a drop in CHr during rhEPO therapy in three differing regimens (group 1, 4 × 300 U/kg; group 2, 3 × 400 U/kg; group 3, 2 × 600 U/kg) and interpreted this as an expression of iron-limited erythropoiesis. The decrease in CHr was largest in group 1 and lowest in group 3. In other words, when the frequency of administration was increased, the release of reticulocytes with lower hemoglobin content was also increased, even though the total dose was similar. Further, Brugnara et al. [9] observed a strong negative correlation between baseline ferritin and CHr course. Since, in our study, the preoperative change in CHr was independent of all iron parameters (baseline values and preoperative change) and did not correlate with the preoperative change in hematocrit/hemoglobin, it is doubtful whether this parameter would be appropriate for identifying iron-limited erythropoiesis, or whether the CHr drop can be avoided by intravenous iron administration during our rhEPO treatment regimen. The absence of the aforementioned correlations suggests that, irrespective of the iron parameters during high-stimulation erythropoiesis, the release of reticulocytes with low hemoglobin content increases after a certain period of treatment (in our trial after 1 week) in all patients, and that reticulocyte maturation and probably hemoglobin formation are shifted from the bone marrow towards the peripheral blood in line with the dose-proportional stimulation of erythropoiesis by rhEPO. Since CHr only began to fall in the second week of treatment it is estimated that only half of all erythrocytes formed during epoetin beta therapy are covered by this drop in CHr (approximately 7% of all erythrocytes present at end of treatment). Thus the reduction in CHr is of minimal significance in our particular treatment regimen. During long-term treatment, however, e.g., for renal anemia, this reduction may be of greater importance in view of a potential functional iron deficiency.

Since CHr did not change in parallel with the HR fraction and since both parameters showed no correlation in the first week, there is nothing to suggest that HR cells possessed a lower hemoglobin content. The negative correlation between the preoperative change in HR fraction

and transferrin saturation at baseline and the preoperative change in iron could signify that, in patients with functional iron deficiency, erythropoiesis is more strongly stimulated by rhEPO-iron combination therapy, and that the likelihood of the omission of maturation stages of erythropoiesis is greater than in patients without iron deficiency. The absence of any correlation between preoperative change in the HR fraction, on the one hand, and preoperative change in hematocrit/hemoglobin and CHr on the other, suggests that HR reticulocytes probably possess a normal hemoglobin content and cannot therefore serve as a parameter for identifying iron-limited erythropoiesis in our rhEPO treatment regimen.

The postoperative hematocrit kinetics also reflect the changes in plasma volume and the shifts in perioperative fluid balance. Erythropoietic activity in both patient groups can be analyzed more objectively by reticulocyte flow cytometry. The slow and flat postoperative increase in reticulocytes in the placebo patients shows that perioperative erythropoiesis stimulation was much less marked than in the epoetin beta group preoperatively. The weaker erythropoiesis stimulation has no significant qualitative effect on the erythropoiesis stages preceding the reticulocytes, with the CHCMr and MCVr remaining unchanged.

The reductions in HR and MCVr and the rise in CHCMr show that the rhEPO effect is no longer present in the epoetin beta patients. Nevertheless, the reticulocytes were still much higher in the epoetin beta group than in the placebo group up until the fifth postoperative day. The postoperative reticulocyte release and the slight, delayed drop in hematocrit at this time was no longer due to any direct epoetin beta effect, since the final dose had been administered 2 days before operation. The half-life of intravenously administered rhEPO lasts from 4–8 hr, with EPO levels returning to normal after 48 hr [19]. The more likely conclusion is that endogenous EPO is formed as a result of the above-mentioned perioperative stimuli, leading to subsequent erythroblast maturation and the release of preformed reticulocytes and to a rapid postoperative normalization of hematocrit due to rhEPO therapy [5,20].

The postoperative drop in CHr results, on the one hand, from the rise of active postoperative ferritin synthesis connected with increased binding of iron and, on the other (only in the epoetin beta patients), from the preoperative epoetin beta therapy. The competing demands of the synthesis of hemoglobin and ferritin for iron are apparent in the placebo group by the onset of a reduction in CHr in the immediate postoperative period. Up until the second postoperative day the slightly reduced CHr in epoetin beta patients is accompanied by the presence of reticulocytes most recently formed preoperatively. This drop is of minimal importance for the total red cell population postoperatively.

The absence of postoperative correlations between the hematocrit in the bypass phase, perioperative blood loss, and the fall in hematocrit/hemoglobin up until the second postoperative day, on the one hand, and the postoperative kinetics of the reticulocyte parameters on the other, shows that multifactorial influences are acting here on erythropoiesis stimulation and response.

CONCLUSIONS

The reticulocyte parameters measured by flow cytometry permitted an objective analysis of erythropoietic activity during treatment with epoetin beta and in all patients postoperatively. Further trials in various types of rhEPO therapy (e.g., long-term treatment in patients with renal anemia or anemia of acquired chronic disease, in patients with autologous blood donation, in anemic cancer patients) should investigate whether the rise in HR fraction and the fall in the CHr in stimulated erythropoiesis are indicators of the premature release of reticulocytes into peripheral blood, or rather of iron-limited erythropoiesis, or a combination of both. Thus, any adjustments of iron medication (e.g., intravenous iron therapy) and rhEPO treatment regimen (e.g., magnitude of single and total dose, frequency of administration) would be possible according to the kinetics of these parameters in order to avoid a functional iron deficiency and to optimize the hematocrit/hemoglobin increase.

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